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INTERACTIONS BETWEEN INNATE IMMUNE EFFECTORS AND MULTIDRUG RESISTANT BACTERIA

Harpa Karadottir

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Interactions between innate immune effectors and multidrug resistant bacteria

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Harpa Káradóttir

Principal Supervisor:

Associate Prof. Peter Bergman

Karolinska Institutet

Department of Laboratory Medicine

Division of Clinical Microbiology

Co-supervisor(s):

Prof. Birgitta Agerberth

Karolinska Institutet

Department of Laboratory Medicine

Division of Clinical Microbiology

Prof. Christian Giske

Karolinska Institutet

Department of Laboratory Medicine

Division of Clinical Microbiology

Dr. Maarten Coorens

Karolinska Institutet

Department of Laboratory Medicine

Division of Clinical Microbiology

Opponent:

Prof. Niels Frimodt-Moller

Rigshospitalet, Copenhagen

Department of Clinical Microbiology

Denmark

Examination Board:

Prof. Mikael Rhen

Karolinska Institutet

Department of Microbiology, Tumor and Cell biology

Associate Prof. Annelie Tjernlund

Karolinska Institutet

Department of Medicine

Center for Molecular Medicine

Associate Prof. Johan Tham

Lund University

Department of Translational Medicine

Division of Clinical Infectious Medicine

ABSTRACT

Antibiotic resistance is an increasingly difficult problem in the clinic, where conventional antibiotics are failing, and new alternative solutions are in high demand. Infections caused by Gram-negative bacteria with multi drug resistance (MDR) mechanisms are increasing globally, and treatment options are limited. Plasmids encoding β-lactamases spread easily between bacteria, and the overuse of antibiotics select for MDR strains. β-lactamases are either serine-β-lactamases that are inhibited by certain β-lactamase inhibitors, and metallo-β-lactamases (MBLs), which are more difficult to inhibit with drugs. The current approach to fight MDR pathogens has mainly focused on finding β-lactamase inhibitors to use in combination with conventional antibiotics in the clinic.

The overall aim of this thesis was to study the role of the cellular micro-environment and the importance of the innate immune system for antibiotic resistant bacterial infections.

In **Paper I** we hypothesized that human cells secreted factors that could impair β-lactamase function and thus restore antibiotic susceptibility in resistant bacterial isolates. We found that thiols produced by the cells acted as zinc chelators that inhibited the degradation of cephalosporin antibiotics in VIM-1 producing *K. pneumoniae*. Notably, free thiols in urine samples had the same effect, suggesting that the environment at the site of infection can be highly important for antibiotic susceptibility and possibly also for the effect of antibiotic treatment in a clinical situation

In **Paper II**, we hypothesized that induction of innate effector molecules would reduce intracellular growth of MDR *K. pneumoniae* and exert synergistic effects with conventional antibiotics. We tested this by infecting human macrophages with MDR *K. pneumoniae*. Notably, induction of innate immunity in these cells resulted in improved intracellular killing of MDR *K. pneumoniae*. The inducers were combined with traditional antibiotics, which resulted in an additive killing effect. The data suggests that inducing innate immune effectors can be an effective alternative or addition to conventional treatments in infections caused by MDR *K. pneumoniae.*

Finally, in **Paper III,** we tested the hypothesis that ESBL *E. coli* would be more susceptible to innate effectors compared to non-ESBL isolates. The ESBL producing isolates had lower survival in serum and whole blood than non-ESBL isolates, suggesting a biological cost for resistant isolates. *In vivo* studies with zebrafish embryos showed that the non-ESBL isolates killed the embryos more efficiently than ESBL isolates. The biological cost was not related to the ESBL plasmid *per se* as shown by experiments where the ESBL plasmid was transferred from a clinical isolate to a neutral background in non-resistant *E. coli*.

Together, this thesis has highlighted the importance of considering the micro-environment at the site of infection, which may determine the effect of antibiotics. Next, I have shown that induction of innate immune effectors could be an alternative or additive treatment option for infections caused by MDR *K. pneumoniae*. Finally, I present data showing that ESBL *E. coli* are more susceptible to innate effectors than non-ESBL *E. coli.*

LIST OF SCIENTIFIC PAPERS

I. **Karadottir, H**. Coorens, M. Liu, Z. Wang, Y. Agerberth, B. Giske, C. Bergman, P. Klebsiella pneumoniae expressing VIM-1 Metallo-β-Lactamase is resensitized to cefotaxime via thiol-mediated zinc chelation. *Infect. Immun.* **88**, (2020) doi: 10.1128/IAI.00756-19

II. Rekha, R. S. **Karadottir, H**. Ahmed, S. Gudmundsson, GH. Agerberth, B. Bergman, P. Innate effector systems in primary human macrophages sensitize multidrug resistant *Klebsiella pneumoniae* to antibiotics . *Infect. Immun.* (2020) doi:10.1128/iai.00186-20.

III. **Karadottir, H**. Ahmed, S. Rajer, F. Coorens, M. Fröding, I. Giske, C. Sandegren, L. Bergman, P. ESBL-producing *E. coli* are more Susceptible to Innate Effector Systems than Non-ESBL *E. coli*. Submitted manuscript Sept. 2020.

OTHER RELATED PUBLICATIONS

1. **Karadottir, H**., Kulkarni, N. N., Gudjonsson, T., Karason, S. & Gudmundsson, G. H. Cyclic mechanical stretch down-regulates cathelicidin antimicrobial peptide expression and activates a pro-inflammatory response in human bronchial epithelial cells. *PeerJ* **3**, e1483 (2015) doi: 10.7717/peerj.1483.

2. Adwani, S. Padhi, A. **Karadottir, H**. Mörman, C. Gräslund, A. Végvári, Á. Johansson, J. Rising, A. Agerberth, B. Bergman, P. Citrullination alters the antibacterial and anti-inflammatory functions of the antimicrobial peptide canine cathelicidin K9CATH *in vitro*. Manuscript to be submitted in 2020.

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LIST OF ABBREVIATIONS

AB	Antibiotic
ANOVA	Analysis of variance
AMPs	Antimicrobial peptides
ATCC	American tissue culture collection
ATG	Autophagy related gene
BPI	Bactericidal/permeability increasing protein
CAMP	Cathelicidin antimicrobial peptide (encoding LL-37)
CAT	Catalase
CFU	Colony forming unit
CHL	Chloramphenicol
CYBB	Cytochrome B-245 Beta chain
DAP	Diaminopimelic acid
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
ESBL	Extended-spectrum β-lactamase
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPx-3	Glutathione peroxidase 3
hBD	Human β-defensin
HDAC	Histone deacetylase
HDACi	Histone deacetylation inhibitor
HDT	Host directed therapy
IFNγ	Interferon gamma
IL-4	Interleukin 4
IMP	Active on imipenem

KPC	*Klebsiella pneumoniae* carbapenemase
LB	Luria Broth
LCN2	Lipocalin-2
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MAMPs	Microbial-associated molecular patterns
MBL	Metallo-β-lactamase
M-CSF	Macrophage colony-stimulating factor
MDR	Multi drug resistant
MHB	Mueller Hinton Broth
MRSA	Methicillin resistant *Staphylococcus Aureus*
Mtb	*Mycobacterium tuberculosis*
NADPH	Nicotinamide adenine dinucleotide phosphate
NDM	New-Delhi metallo-β-lactamase
NFκB	Nuclear factor κB
NLR	Nod-like receptor
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NOX	NADPH oxidase
OD	Optical density
OXA	Oxacillin resistant
PBA	Phenylbutyric acid
PBMCs	Peripheral blood mononuclear cells
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PDR	Pan-drug resistant
PRR	Pattern recognition receptor
ROS	Reactive oxygen species

RPMI	Roswell Park Memorial Institute medium
RT-PCR	Real time polymerase chain reaction
SBL	Serine-β-lactamase
siRNA	Small interfering ribonucleic acid
SPS	Sodium polyanethol sulfonate
TLR	Toll like receptor
VDR	Vitamin D receptor
VIM	Verona-integron encoded metallo-β-lactamase
WHO	World Health Organization
WT	Wild type
XDR	Extensively drug resistant

1 INTRODUCTION

Bacterial strains may acquire resistance at a fast rate, and MDR strains have rapidly circumvented the effects of many available antibiotics. There is an undisputable need for a better understanding and improved treatment of infections caused by antibiotic resistant bacteria, which have become problematic in hospital settings, the community and environment in many parts of the world. The population most affected by antibiotic resistant bacterial infections are the elderly and immunocompromised, resulting in a higher risk for sepsis and death and making the hospital setting an important arena for the rise in resistant bacterial strains. This is not only affecting survival rates of patients, but is also associated with a high cost increase for the health care sector, where morbidity, mortality and lengths of hospital stays increase[1]. It is estimated that the number of annual deaths caused by antimicrobial resistance will reach 10 million in the year 2050. In comparison, the number of deaths caused by road traffic accidents, cancer and diabetes combined are expected to be around 11 million annually[2,3,4].

Hospital acquired infections come in fifth as the leading cause of death in the acute care, where the at-risk population is mainly infants, the elderly and immunocompromised patients. Within the hospital setting, bacteria can spread from health care workers to patients as well as between patients. Importantly, opportunistic bacteria can use plastic devices, such as catheters and ventilators as scaffolds for their growth and may thus cause disease. It is important to take preventive measures to inhibit the transmission between patients with proper hand hygiene, which has been shown to be effective. With the rise of antibiotic resistance, hospital-acquired infections are becoming increasingly difficult to handle[5]. The burden of disease varies for different bacteria, where extended spectrum cephalosporin resistant Gram-negative bacteria, together with carbapenem resistant Gram-negative bacteria constitute a special and emerging problem[1].

The ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinobacter baumannii, Pseudomonas aeruginosa and Enterobacter spp*) are of high interest because of their ability to become resistant to our available antibiotics. In recent years, the level of threat has shifted from the Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) to Gram-negative β-lactamase producing strains. From that category the World Health Organization (WHO) published a list of bacteria that are considered a priority and in urgent need for new antibiotics; the Gram-negative *E. coli, K. pneumoniae* and *P. aeruginosa* are in priority 1. The field of antibiotic resistance has mostly focused on the bacterial pathogens and mechanisms of resistance within the bacteria[6], while the contribution of host immunity has been overlooked and understudied. This thesis will focus on two highly relevant groups of antibiotic resistance mechanisms, the extended spectrum β-lactamases (ESBL) and carbapenemases and how bacteria that can acquire these resistance mechanisms interact with innate immune factors contributing to the initial responses and killing of bacteria.

1. INNATE IMMUNE DEFENSES

For bacteria to establish infections, a first step is to circumvent the efficient and thorough innate immune system. The bacteria need to enter from the environment, through the mucosal and epithelial barrier and into the bloodstream to cause an infection, commonly through the digestive tract or the urinary tract. For the bacteria to enter through the epithelial barrier into the bloodstream, there is a need to overcome obstacles, such as antimicrobial peptides (AMPs), the mucosal barrier, tight junctions, phagocytes, and when it comes to the bloodstream the complement system is important to mediate bacterial killing[7].

1.1 THE MUCOSAL BARRIER SYSTEM

The mucosal barrier that separates our internal organs from the external environment consists of different parts that include, from the outer layer inwards; a layer of commensal bacteria that form the microbiota, a mucus layer that gradually increases in thickness, and the epithelial cell layer. The commensal bacteria exist in symbiosis with the host and can be found in e.g. the gastrointestinal tract, the urinary tract, the lungs and the skin. They are essential for the host because of their direct competition with potential pathogens as well as their production of metabolites, such as butyrate and other short chain fatty acids, that can induce AMP- production in epithelial cells, as well as provide important nutrition for these cells. In addition, the microbiota can influence the function of immune cells in the submucosa, and reciprocally the immune cells can regulate the microbiota, keeping a controlled environment without inflammation. Underneath the microbiota, pathogens encounter a mucus layer consisting of a thick mucus (mucin proteins and lipids) produced by goblet cells in the epithelial layer. The epithelial layer itself is a network connected via tight junctional structures to prevent the invasion of microbes. If the pathogen succeeds to enter through the epithelial barrier, it will encounter an army of phagocytic cells that reside in the connective tissue (Figure 1)[8]. Both epithelial cells and immune cells express pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs) and cytoplasmic NOD-like receptors (NLRs). These receptors recognize conserved microbe-associated molecular patterns (MAMPs), which upon binding cause structural changes to the receptor complex that initiates intracellular mechanisms, such as the NFκB pathways that are important for initiating inflammatory responses. Depending on the type of MAMP sensed by the receptor, different pro-inflammatory states can be regulated. If a bacteria reaches or breaches through the epithelial cell layer, the PRRs sense the different MAMPs, such as flagellin, peptidoglycan and lipopolysaccharide (LPS). Once cells are activated through their PRRs, they react by either communication through cytokines with other immune cells, or initiate direct killing by antimicrobial effectors in immune cells[9].

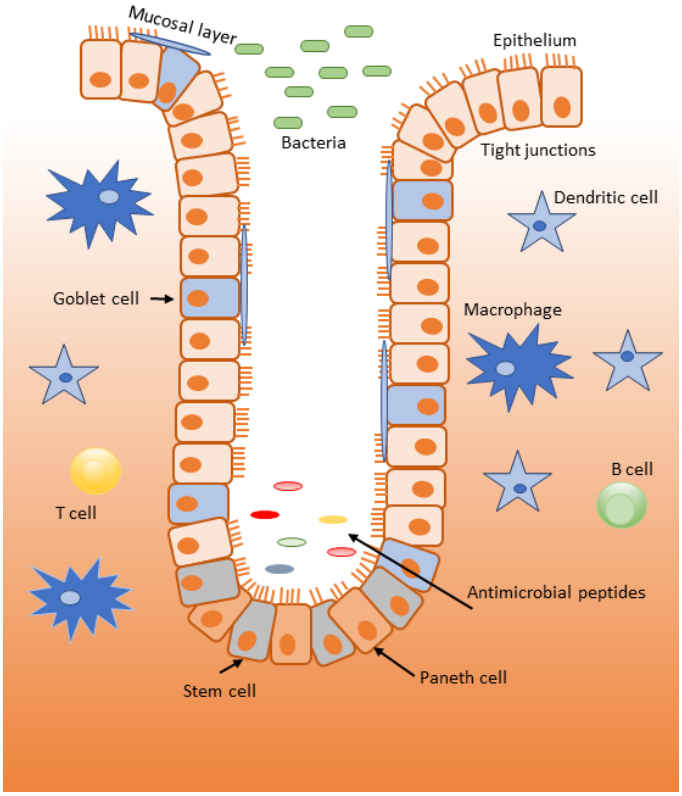


Figure 1. The mucosal barrier. *The mucosal barrier is regulated by cross talk between the microbiota, epithelial cells and immune cells. A gradient of mucus prevents the commensal bacteria to come in contact with the cells, keeping a homeostasis without inflammation. Picture adapted from Wu et al*[10].

1.2 ANTIMICROBIAL PEPTIDES

AMPs are recognized for their potent bactericidal activity and play an important role in the first line of defense against invading bacteria. They are expressed in both epithelial cells and leukocytes, such as macrophages and neutrophils. AMPs are amphipathic with a net positive charge as well as a hydrophobic side. Their antimicrobial mechanism of action is thought to involve an initial electrostatic interaction with lipids in the bacterial outer membrane or cell wall and a subsequent secondary hydrophobic contact that disrupts the membrane, leading to bacterial lysis[4]. AMPs can also have additional functions not directly related to their antimicrobial activity, such as chemotaxis, anti-tumor activity, stimulation of cell proliferation, LPS neutralization and degranulation of mast cells[11,12].

AMPs can be subdivided in different families, with the major families in mammals being the cathelicidins and the defensins. The defensins are further characterized into two families, α and β-defensins, that are similar in secondary structure (small and cysteine rich), but lack homology in amino acid sequence[11]. LL-37 is the only human cathelicidin and its expression can be induced by different stimuli and molecules. For example, vitamin D_3 has a major role in the

regulation of LL-37 expression[13]. Additional known inducers of LL-37 are butyrate (abundant in the gut)[14] and its synthetic analogue phenylbutyric acid (PBA)[15] as well as Entinostat[16], that function as histone deacetylase inhibitors (HDAC inhibitors). Importantly, while the identification of novel AMP inducers is interesting from a biological point of view, it has also given rise to the development of host directed therapy (HDT) to fight infections, an innovative potential therapeutic approach to counter bacterial infections through stimulation of the host immune system by exogenously applied small-molecular compounds[13].

1.3 ANTIMICROBIAL PROTEINS

In addition to AMPs, antimicrobial proteins (defined as mature and active proteins larger than 100 amino acids) are present in humans, which can exert direct or indirect killing of bacteria[19]. For instance, lysozyme is an enzyme abundantly found in tears, saliva and mucus. It catalyzes the hydrolysis of the peptidoglycan in the bacterial cell wall, resulting in lysis of the bacteria. Gram-positive bacteria are highly susceptible to the activity of lysozyme[20], whereas Gram-negative bacteria are protected by LPS of the outer membrane. Other antimicrobial proteins are for example lactoferrin, calprotectin and lipocalin-2 (LCN2). LCN2 is a 25 kDa secretory glycoprotein that was originally purified from neutrophils[21], but is also expressed by epithelial cells and is upregulated upon stress, such as gastrointestinal damage, bacterial infection or inflammation[22]. The antimicrobial activity of LCN2 comes from its ability to bind and neutralize iron bound siderophores, which allows it to compete for available iron and prevent bacterial iron acquisition that is essential for the bacterial growth[23]. Siderophores are produced by some bacterial strains, including *Klebsiella pneumoniae,* and are secreted to acquire iron from the environment[24].

1.4 CELLS OF THE INNATE IMMUNITY

One important component of innate immunity are phagocytes that directly attack or engulf invading bacteria. They are found in tissues or circulating in the bloodstream, where they search for foreign invaders. Professional phagocytes include macrophages, neutrophilic granulocytes and dendritic cells. Macrophages can be either tissue resident or differentiate from blood derived monocytes upon entering inflamed tissues and phagocytose microbes that have penetrated the mucosal barriers. Upon activation, these macrophages also promote secretion of cytokines and chemokines that recruit more immune cells to the site of infections. Neutrophils contain granules filled with various antimicrobial factors, including AMPs. Once a bacterium is phagocytosed, these granules fuse with the bacteria-containing phagosome, promoting direct killing of the pathogen[25]. Another part of the antimicrobial arsenal of phagocytes is reactive oxygen species (ROS). The release of ROS is largely depending on external factors, such as stress and the presence of microbes, and can either be considered beneficial as an immune response, or harmful in the case of excessive stress[26,27]. The general ROS production and release is highly regulated, and mostly generated in the mitochondria. The cells maintain ROS

at low levels and exhibit a steady-state to reach a balance of generation and elimination of the free radicals[28]. In phagocytes, ROS are mainly produced by a multiprotein electron transferase NADPH oxidase (NOX2) complex. The produced ROS can oxidize proteins, DNA and carbohydrates, resulting in killing of pathogens[29].

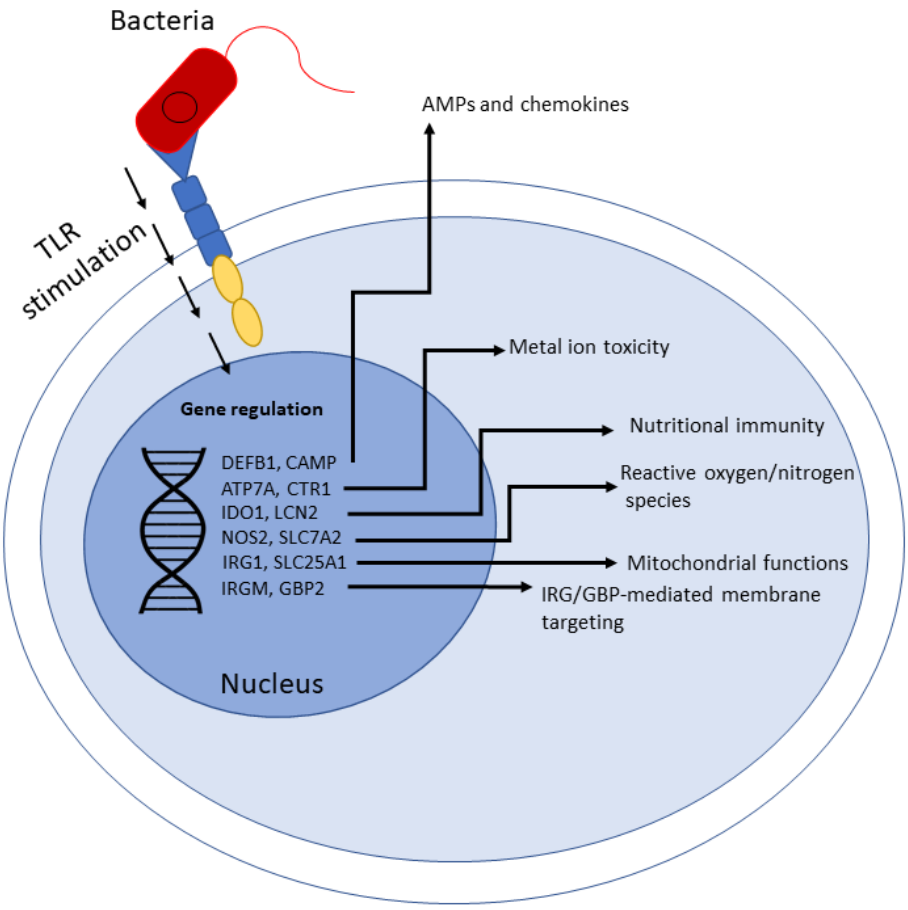


***Figure 2. TLR activation results in an innate immune respnse**. A bacterial component is recognized by a TLR-receptor on the cell surface, resulting in regulation of genes that promote an innate immune response. Picture adapted from Stocks, et al (2018)*[30].

Finally, the dendritic cells serve a special function among phagocytes and provide a link between the innate immune system and the adaptive immune system through antigen presenting abilities. They can activate T cells by presenting phagocytosed antigens and initiating antigen specific immune responses[31].

1.4.1 MACROPHAGES

Although all of the above mentioned cell types are important for the innate immune system, I have mainly worked with macrophages as a model system in this thesis. Monocyte derived macrophages (MDMs) have previously been catagorized into two types; M1 and M2 depending on the polarization and inflammatory state of the cells. The fundamentals of the characterization is based on the observation that macrophages either kill pathogens (M1 type), while other macrophages contribute to tissue repair (M2 type)[32], in other words pro-inflammatory (M1) or anti-inflammatory (M2) responses. Although it is convenient to group the macrophages into two distinct groups, this characterization may represent a simplistic view[33]. *In vitro* macrophages can be induced by certain compounds that differentiate them into M1 (GM-CSF, INF_{γ} and LPS) or M2 (M-CSF and IL-4) macrophages. However, the situation *in vivo* represent a much more complex system [34]. The M1/M2 paradigm can be considered as a spectrum, where certain stimuli also can cause the cells to differentiate outside of the spectrum. Since macrophages are considered specific to the organ they reside in and have the ability to alter their location-based differentiation, it can be concluded that the double-ended M1-M2 spectrum can be useful for simple clarification of the inflammatory responses, but not enough to characterize the cell type[33,35].

Macrophages can detect pathogens through various pattern recognition receptors that sense common and conserved components of pathogens, receptors such as the toll-like-receptors (TLR). TLRs can bind to microbial components, where the most extensively studied antigen-receptor binding is the lipopolysaccharide (LPS) binding to TLR-4. Upon binding, a signaling cascade initiates an innate immune response leading to cell activation through the NFκB pathway, resulting in a pro-inflammatory response[36].

When a macrophage is activated, different forms of defense mechanisms can be initiated to eliminate pathogens in the extra- or intracellular compartments. Cytokines and chemokines are secreted to activate and recruit more immune cells, as well as AMPs and ROS for direct killing of the pathogen. When the pathogen is phagocytosed, intracellular elimination mechanisms occur, where autophagy is an important contributing factor in the pathogen elimination. The pathogen is transfered by several regulated steps to the autophagosome and fuses with the lysosome, where autophagy can promote the addition of antimicrobial peptides and ROS to the lysosome to enhance the killing. This form of autophagy is regulated by genes downstream the NFκB pathway, such as the ATG genes (ATG-14, ATG-5, Beclin-1). Autophagy is particularly important for the control of intracellular bacterial infections. However, certain virulent bacteria, including *Mycobacterium tuberculosis* (Mtb), has developed mechanisms to evade autophagy[37,38].

1.5 ANTIMICROBIAL FACTORS IN THE BLOOD

The blood has many different antimicrobial components. A potent antimicrobial response is the complement mediated killing of bacteria in serum. When bacteria enter the bloodstream and their antigens are detected, the activation of the complement system can occur through three different pathways called the Classical pathway, the Alternative pathway and the Lectin pathway. Activation of these pathways leads to the serial activation of complement components where activation of specific complement components results in opsonization of the bacteria with C3 fragments that bind to the bacterial surface and label it for more efficient phagocytosis. Activation of the complement system can also kill bacteria directly through the formation of a membrane attack complex (MAC). A variety of protein fragments are involved in the activation of the MAC and the destruction of the bacteria is through lytic effects[25,26]. *In vitro*, the complement pathway can be inhibited by either a physical (heat) or chemical (sodium polyanethole sulfonate, SPS) inactivation. SPS is currently used as an anti-coagulant and complement inhibitor in blood cultures in the clinical setting, and blocks the activation of the classical and alternative complement pathways, whereas the lectin pathway is not inhibited[41].

Not all bacteria are equally sensitive to direct complement killing, where some strains can be serum resistant and thus thrive in human blood. Depending on the surface of the bacteria, the susceptibility to serum can vary[42,43]. In addition to the complement system, other antimicrobial factors, such as lysozyme, can assist in the killing of bacteria in the blood[20].

E.coli is the most common cause of bloodstream infections, where strains with resistance to serum often express capsules or modified LPS, where a longer LPS structure is associated with serum resistance and initiation of complement-activation is located at a longer distance from the bacterial surface[44]. The blood also contains circulating monocytes and neutrophils that produce antimicrobial agents, including reactive oxygen species (ROS) and nitric oxide (NO) which exhibits potent effects on bacterial survival[29]. A range of antimicrobial peptides and proteins can be found in the blood, mainly within circulating leukocytes and platelets and secreted in response to infection. Lysozyme and phospholipase A2 enhance the lysis of cells and work in combination with the complement system for efficient killing[45,46]. Additionally, LL-37 and bactericidal permeability increasing protein (BPI) neutralize endotoxins and cause direct killing of the pathogen[47,48]. When bacteria evade the bactericidal factors of the blood, bacteremia can develop, which can lead to sepsis, organ failure and sometimes death[44].

2. GRAM-NEGATIVE BACTERIA

Since the discovery of penicillin, methicillin resistance in *Staphylococcus aureus* and vancomycin resistant enterococci, Gram-positive bacteria were considered of most concern. More recently, Gram-negative infections have emerged as the most difficult to treat and cause clinical problems due to bacterial resistance[49]. Gram-negative bacteria allow for a silent and rapid exchange of genes, carrying resistance mechanisms to strains causing community onset and health-care associated infections[50].

2.1 THE OUTER MEMBRANE OF GRAM-NEGATIVE BACTERIA

The outer membrane of Gram-negative bacteria is rich in lipopolysaccharides (LPS). LPS are large lipid molecules consisting of lipid A, an inner and outer saccharide core and a hydrophilic O-antigen. The lipid A acts as an anchor between the outer membrane and the sugar moieties of the LPS molecule and plays a role in stabilizing the bacterial outer membrane with its hydrophobic saturated fatty acyl chains[49]. Depending on the bacterial species, lipid A can present a different extent of acetylation and phosphorylation in different bacteria. In addition, the symmetry (depending on number of acyl chains) of the lipids can vary. The degree of phosphorylation of the lipid A can determine the net charge of the membrane (at specific areas or overall), giving a negative charge to the membrane[49]. The net charge is an important factor for the host immune response, including cationic AMPs that interact with the negatively charged areas, resulting in breakdown of the membrane[49]. The second part of the LPS is the inner core, which consists of sugars and is more highly conserved among bacterial species than the other two parts. The inner core is important for cell viability and has certain antigenic properties when the LPS does not contain any O-antigen[51]. The O-antigen consists of polysaccharides and stand out from the bacterial membrane into the extracellular environment. This glycan can vary in length and consists of repeating sugar units and can either be present or lacking in the LPS structure[52]. When the O-antigen is present, the LPS is referred to as smooth LPS (S)-form, while LPS lacking O-antigen is called rough LPS (R)-form. The O-antigen plays an important role in pathogenicity and virulence of bacteria and is a highly immunogenic antigen and recognized by the host immune system. Rough LPS leaves the cell more permeable and thus more susceptible to antimicrobial agents and environmental factors, such as stress, which indicates that changes in LPS can have a great impact on the bacteria[51]. The outer membrane of Gram-negative bacteria is important for antimicrobial resistance properties, especially in bacteria such as *K. pneumoniae* that can also be coated with a thick polysaccharide capsule outside of the outer membrane and as a result is less affected by AMPs, phagocytosis and complement mediated killing in human serum[40,53].

2.2 ENTEROBACTERALES

Two of the most problematic Gram-negative bacteria with regard to antibiotic resistance belong to the family of *Enterobacterales* (formerly called *Enterobacteriacae*). They are non-spore forming, generally ferment glucose, reduce nitrate and can be either motile with flagellas or non-motile. The *Enterobacterales* are found in nature and constitute a part of our natural microbiota but can also become pathogenic and cause disease. In part, this depends on their ability to transfer and acquire specific virulence factors between strains. Numerous virulence factors can be transmitted between bacteria on mobile genetic elements; plasmids, which can result in highly pathogenic strains. The pathogenic strains have often acquired special adhesion or colonization attributes (pili), which help them to colonize places they usually do not reside in (e.g small intestines or urethra). These bacteria can also produces enterotoxins[54]. Similarly, transmission of genetic material between strains can lead to antibiotic resistant strains, where the overuse of antibiotics selects for these strains to survive and cause disease. Two members of the *Enterobacterales* family are *E. coli* and *K. pneumoniae*, which are common causes of bacterial infections, especially in the hospital setting[1]. Their ability to easily acquire resistance is of high concern today, with emergence of strains expressing extended-spectrum β-lactamases (ESBL) and carbapenemases[55]. Bacterial species, such as *K. pneumoniae* and *E. coli* have developed a protective mechanism by producing several iron-binding molecules, called siderophores (e.g enterobactin, salmochelin, yersiniabactin). Since iron is important for bacterial growth, siderophores constitute an excellent target for host immunity. Consequently, the host has developed ways to restrict access to iron by using LCN-2, which binds to siderophores and thus block iron-uptake by bacteria. It has been shown that LCN-2 is necessary for protection of the lung against *K. pneumoniae* and *E. coli*[24,56].

E. coli normally resides in the gastrointestinal tract and coexist with humans throughout the lifespan without any complications. Commensal *E. coli* is considered opportunistic and only cause infection when the gut barrier is breached or in immunocompromised individuals where the balance between the gut microbiota and the immune system is dysregulated. *E. coli* mainly cause gastrointestinal infections, urinary tract infections and sepsis/meningitis[54].

Similarly to *E. coli*, *K. pneumoniae* is carried by humans, mainly in the GI-tract, but can also be found in the upper respiratory tract and skin. The bacteria are rod shaped, non-motile and can have a variety of capsules that can be serotyped (K-typing) and are associated with virulence factors[57]. Infections caused by *K. pneumoniae* are mainly hospital acquired and presents as pneumonia, urinary tract infection or sepsis, mainly in the immunocompromised, neonates or the elderly[58]. However, hypervirulent strains can cause community acquired infections in previously healthy humans. These strains often belong to certain capsule serotypes, such as K1 and K2[59]. In addition to K-typing, O-antigens are also used to type the bacteria, where O1 serotype is highly prevalent in clinical isolates. The capsule can protect against host immune responses, including phagocytosis, complement-mediated killing and AMPs[57].

3. ANTIBIOTIC RESISTANCE

The first antibiotic was discovered by Alexander Fleming in 1928, isolated from the fungus *Penicillium notatum*[60]. Since then, a range of additional antibiotic-classes have been discovered, including cefalosporins, carbapenems, macrolides, and quinolones to mention the most important. However in recent years, with the arising problem of antibiotic resistance, inventing new classes of antibiotics has proven difficult and bacteria have rapidly gained resistance to the few novel antibiotic drugs that have been introduce to the market[61].

Antibiotics can be categorized in different ways according to their structure or mechanism of action. The classification by target includes antibiotics that interfere with the bacterial cell wall, the protein synthesis and DNA synthesis. β-lactams comprise the largest group of antibiotics and have been shown to be very potent against bacteria for decades. All β-lactams have a conserved ring structure with various additional chemical groups for a variety of functions. The four membered β-lactam rings are responsible for the antibacterial effect by binding as a substrate to the Penicillin Binding Protein (PBP) on the bacterial wall, that cross link the peptidoglycan in the genesis of the cell wall, thus impairing cell wall synthesis for replicating cells resulting in lysis of the bacteria. The β-lactams have multiple sub-classes that include penicillins, cephalosporins, carbapenems and monobactams, where carbapenems are reserved for the most complicated Gram-negative infections. Multiple resistance mechanisms exist, such as active efflux, reduction of bacterial membrane and cell wall permeability, altering transpeptidases, and the enzymatic inactivation of β-lactam antibiotics by β-lactamases[62]. Today, resistance mechanisms have been discovered in bacteria that can affect every type of available antibiotics, and a special concern is the β-lactamase producing Gram-negative bacteria. In particular, the resistance to carbapenems is problematic and widespread, by virtue of the expression of carbapenemases[61]. When a bacteria is resistant to multiple antibiotic types, the terminology MDR (Multi drug resistance) is used, while XDR and PDR refer to extensively drug-resistant and pandrug- resistant (pan referring to all antibiotics), respectively[63].

3.1. HORIZONTAL GENE TRANSFER

Bacteria have the ability to exchange genetic material (plasmids) through different mechanisms, which is crucial for their evolution[64]. The three main mechanisms for horizontal gene transfer are transformation, transduction and conjugation. Transformation is the process where foreign DNA from the environment is incorporated into the bacterial genome, often from either lysed cells or actively secreted material[65]. Transduction occurs through bacteriophages, where the phages replicate inside the bacterial cell and bacterial DNA can be incorporated in the phage capsids[66]. The third mechanism is conjugation, an important factor in antibiotic resistance. The genetic material is transferred from a host cell to a recipient cell through a passage or a secretion system, where the system is usually expressed by proteins on the plasmid[67]. Antibiotic resistance genes can be carried on plasmids and transferred between bacteria via horizontal gene transfer, most commonly through the third mechanism, plasmid

conjugation. This results in a complicated fight against antimicrobial resistance, where one bacterium can spread a resistance mechanism easily to surrounding bacteria[68,69].

3.2. β-LACTAMASES

The emergence of antibiotic resistance is not recent, but represent ancient events designed in nature that helped bacteria or fungal species to survive in a hostile environment full of competing species secreting antibiotic compounds. With the use of antibiotics, strains that did not contain any defenses against antibiotics were eliminated, while the bacteria that contained resistance mechanisms survived, and in that way were selected to survive and spread[70]. β-lactamases are enzymes produced by bacteria that can cleave the four membered ring of β-lactams, resulting in the inactivation of the antibiotic. This resistance mechanism is of special concern, where the gene expression of these enzymes can be transported between bacteria on plasmids, allowing for a fast spread. These enzymes have been classified by the Ambler system into different groups; A, B, C and D β-lactamases based on the molecular size and similarities in active sites of the enzymes[71]. Older classifications were made from the type of hydrolysis by the active site of the enzyme and how the enzymes cleave the antibiotics; serine-β-lactamases and metallo-β-lactamases[61]. Classes A, C and D all belong to the group of serine-β-lactamases (SBL) while class B belongs to metallo-β-lactamases (MBL)[62].

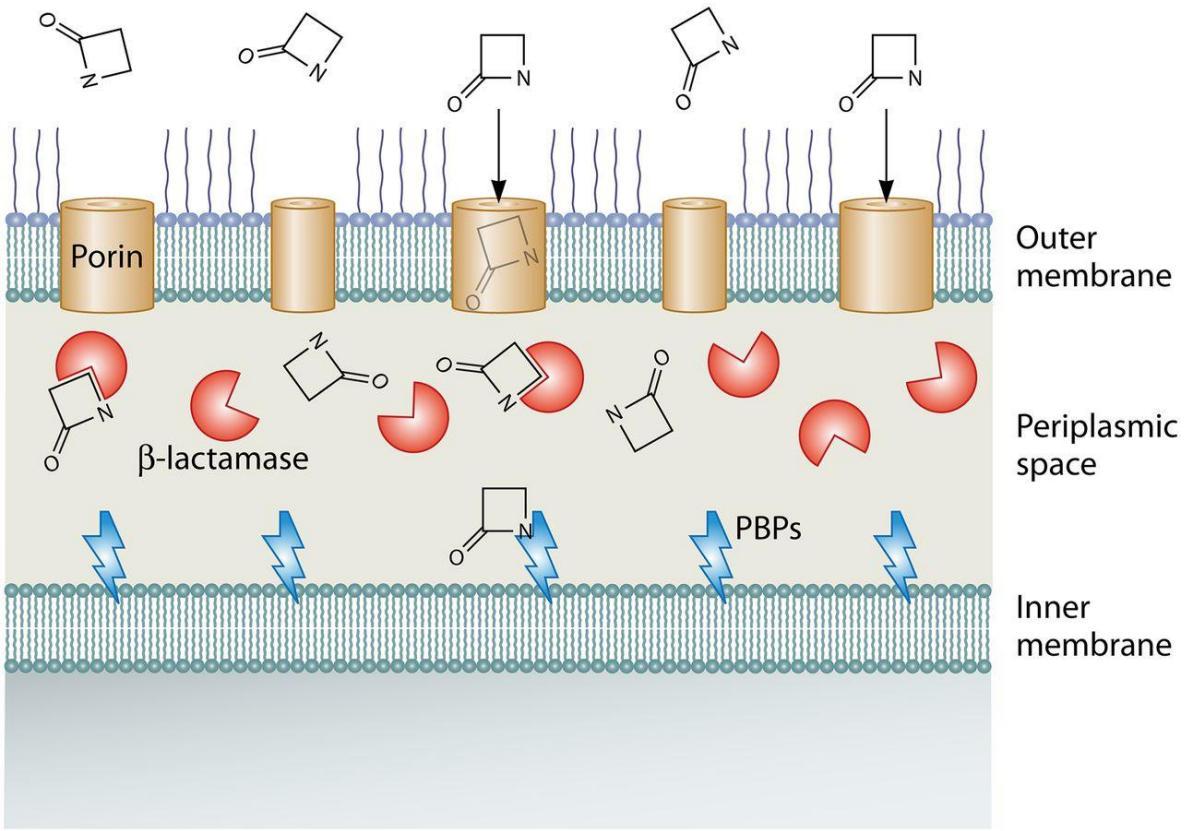


Figure 3. The activity of β-lactamases*. β-lactam antibiotics bind to the penicilling-binding-protein (PBP) on the bacterial membrane, and impair the cell wall synthesis. β-lactamases are produced by the bacteria and cleave the beta-lactam ring of the antibiotics, which prevent them from binding to the PBPs. Figure reused with permission from the publishing journal*[72]

SERINE-β-LACTAMASES This group of β-lactamases have a serine amino acid in the active site of the enzyme that catalyzes the hydrolysis of the amide bond in the four membered β-lactam ring, opening up the structure and preventing it to bind the PBP for inactivation of the cell wall synthesis. The importance of the serine residue is the interaction with the β-lactam, forming a covalent serine-bound acyl intermediate. The different groups of serine-β-lactamases show a conserved structure of the active site, suggesting a common origin in evolution, and are considered to go back 2 million years[73]. Various serine-β-lactamases have been identified and they are becoming more visible in health care, including extended spectrum β-lactamases (ESBL) that are known to break down cephalosporins, *Klebsiella pneumoniae* carbapenemase (KPC) and the oxacillinase (e.g OXA) that can hydrolyse carbapenems and penicillins respectively. The ESBLs have been shown to be inhibited by clavulanic acid, however the ESBL-producing bacteria pose a threat both in community and hospital settings, where outbreaks have been associated with ESBL carrying *E. coli*. A certain virulent variant of ESBL, the CTX-M family, has shown dominance within the ESBL-producing Gram-negative population. With the ESBL-producing isolates becoming more prominent in the hospitals,

antibiotics such as carbapenems, that had been reserved for more complicated infections, were increasingly used. This increased usage has resulted in isolates that have gained multiple β-lactamases (MDR strains), where the combinations of β-lactam antibiotics and clavulanic acid/inhibitors is insufficient. This selection of strains with carbapenem resistance has made various available antibiotics inefficient against infections, and underlines the importance of finding alternative ways of resolving difficult infections[73].

METALLO-β-LACTAMASES The second type of β-lactamases are the metallo-β-lactamases (MBL). The first MBLs identified were the enzymes IMP (Imipenemase-type metallo-β-lactamase) and VIM (Verona Integrated metallo-β-lactamase) that spread within Gram-negative pathogens including Enterobacteriales. More recently, the NDM (New Delhi metallo-β-lactamase) was identified in a patient in Sweden, arriving from India[74]. The different resistance genes are often associated with certain geographic areas, where they are thought to have originated, however the strains are spreading fast through the different parts of the world. As the name indicates, use of metals is essential for the function of MBL enzymes, where either one or two zinc ions are necessary to catalyze the hydrolysis of the β-lactam antibiotics. The hydrolysis does not result in a covalently bound intermediate. The enzyme catalysis begins with the binding of the β-lactam in the active site of the enzyme that contains the metals, where zinc stabilizes the hydroxide ion in the binding site, that with a nucleophilic attack reacts with the carbonyl carbon of the β-lactam, leading to the formation of a tetrahedral-intermediate that results in breaking of the amide bond of the β-lactam. The active sites of these enzymes are not as conserved as the serine-β-lactamases, but have more variety, which indicates that they did not have a common evolutionary origin. The MBLs belong to the Ambler B class of β-lactamases and have constituted a real challenge and have shown to be difficult to inhibit *in vivo*[62].

3.3. β-LACTAMASE INHIBITORS To treat infections caused by resistant bacterial strains, different combinations of antibiotics have been used, and the use of broad spectrum antibiotics has given the strains the opportunity to accumulate different resistance mechanisms and become resistant to multiple antibacterial agents at once. In addition to combining different types of antibiotics, treatments of infections caused by antibiotic resistant pathogens have focused on combining β-lactamase inhibitors with antibiotics[61]. A number of serine-β-lactamase inhibitors have been identified, including the above mentioned clavulanic acid, as well as sulbactam and tazobactam. Other novel inhibitors have been made available, such as avibactam, relebactam and vaborbactam, however none of these show activity agains MBLs[75,76]. The zinc dependency of the MBLs led to the finding that EDTA (divalent metal chelator) could be used as an inhibitor[77]. However EDTA is highly toxic and thus not a viable treatment option as an MBL inhibitor in humans. Nevertheless, EDTA is used in the laboratory to identify MBL-expressing isolates. While the synthesis of MBL inhibitors have not given much success in recent years, multiple studies have shown that chelating zinc can be a viable

option, and currently zinc chelating molecules and other possible inhibitors are being studied[78]. The natural compound aspergillomarasmine A has been shown to inhibit NDM-1 and VIM-2 enzymes through inactivation by Zn^{2+} binding in the active site, and has shown efficiency in *K. pneumoniae* infected mice[79]. Certain compounds, such as thiol containing chemicals, can have zinc chelating abilities and have been proposed as inhibitors in combination with antibiotics[80,81]. The problem that may arise with these inhibitors is the toxic effect of the zinc chelation on the host. Research has thus focused on screening for compounds that do not bind the ions directly, but bind the active site of the metallo-β-lactamase[82]. The most promising inhibitors today are bicyclic boronates that have shown inhibition against both serine-β-lactamases and metallo-β-lactamases[83].

Interestingly, eukaryotic cells have a regulated environment that is also depending on metals, such as zinc. Thus, it would be interesting to search for endogenous zinc chelators that possibly could be used to inhibit bacterial MBLs.

4. BACTERIAL FITNESS

Current research has revealed that the bacterial acquisition of antibiotic resistance mechanism comes at a certain cost of fitness for the bacteria[84]. Studies focusing on competition between susceptible and resistant bacteria have shown that the growth rate is slower when resistance mechanisms are present[85–87]. The current explanation for this is that increased energy is needed for the expression of different efflux pumps and enzymes, which can explain why the acquisition of resistance could alter the energy distribution of the bacterial cell, resulting in a cost of fitness[85]. Strains can also gain resistance against heavy metals (copper, silver, arsenic) in the environment, which might affect the survival in different circumstances[88]. In a study by Schrag et al, the presence of plasmids carrying resistance genes were associated with slower growth, which suggests that there is a cost of fitness for genetic alterations[89], however this cost is strain-specific and the contribution of host defenses against resistant bacteria has been less studied.

The cost of fitness has been efficiently studied on bacterial strains carrying chromosomally encoded resistance genes[90–92]. However plasmid borne resistance has recently been studied to a higher extent, where strains carrying antibiotic resistance encoded on plasmids come with a cost resulting in lower fitness[87,90,93,94]. Methodologically, the biological cost has mostly been studied with competition assays, where strains with selection markers are co-cultured, and the survival recorded. Other studies that include *in vivo* models have recorded similar results, mainly in murine models[84]. The research on biological cost of fitness for resistance acquisition in relation to host recognition and innate immune responses is very limited.

It was concluded from research on strains transfected with an ESBL plasmid versus non-ESBL that no effect was observed on fitness in serum, however the focus of physical attributes of the bacteria was not taken into account[95]. Other studies on *K. pneumoniae* and *E. coli* from urinary

tract infections reported either no effect or beneficial effect of plasmid acquisition, indicating discrepancies and unclear data on serum sensitivity of ESBL and non-ESBL pathogens[96,97].

An important observation concerning the research on cost of fitness is that conditions used to assess growth are often similar to standards developed to test for antibiotic resistance, where bacteria are grown in optimal conditions. These standards are necessary to get comparable results around different laboratories, however it fails to take into account the physiological environment of infection, including innate immune factors. Different studies have addressed this issue, where the culture broth was adjusted by using a more physiological cell culture media (RPMI) supplemented with Luria broth (LB), which proved to have a major impact on experimental outcomes[98]. With new perspectives on host-pathogen interactions, by including the physiological environment of infection, research on bacteria and antibiotic resistance, and antibiotic resistance testing can most likely be improved[99].

An interesting research approach is to spread the focus from direct bactericidal effects to a broader perspective by addressing the problem from multiple directions. Using a monotherapy drug has been shown to result in a rapid acquisition of resistance mechanisms. While it is important to continue the search for more antibacterial drugs, another possible alternative would be to study and utilize pre-existing innate host defenses[100].

5. BLOODSTREAM INFECTIONS AND SEPSIS WTIH FOCUS ON ANTIBIOTIC RESISTANCE

When bacteria enter the bloodstream of patients, referred to as bacteremia, the immune system may react in a dysregulated manner, resultng in sepsis. According to the "Sepsis Definitions Task Force", the term Sepsis-3 is defined as a[101];

"life-threatning organ dysfunction caused by a dysregulated host response to infection" and "Septic shock is a subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality"[101].

To cause an infection, bacteria can cross the mucosal barrier and invade different organs, such as the urinary tract, lungs, skin and soft tissues, which is followed by fever, chills and/or hypertension. *E. coli* and *K. pneumoniae* are two common causes of bacteremia and sepsis[102]. The dramatic increase in antibiotic resistance in those species, makes them highly dangerous infectious agents in a clinical setting, where rapid discovery and treatment is important to avoid morbidity and mortality. The spread of antibiotic resistance varies in different parts of the world, for example, a study by Quan et al, showed that *E. coli* isolated from bloodstream infections exhibited an ESBL-phenotype in 55% of the cases in certain parts of Asia[103]. In the EU the prelevance of ESBL β-lactam resistance in *E. coli* was 14.9% in 2017. Notably, the prevalence of carbapenem resistance in *K. pneumoniae* was over 10%, while carbapenem resistance in *E. coli* is rare in the EU population (<1.6%)[72,104].

6. HOST DIRECTED THERAPY

With fewer available antibiotic treatments, due to increased antibiotic resistance of bacteria, novel and alternative methods need to be explored to resolve bacterial infections. The innate immune system consists of various ancient effectors that attack invading pathogens, and research on the innate immune effector systems has revealed that they can be manipulated and induced by external stimulation using simple drug-like compounds[14,17,18,31,100,105]. While monotherapy, with either antibacterial drugs or innate antimicrobial effectors pose the risk of resistance[106], inducing multiple immune responses through different mechanisms could be a key strategy in the search for alternative methods[100].

Some bacteria have developed mechanisms to alter or suppress the immune responses of the host for its own advantages to establish an active infection. Both epithelial cells and innate immune cells have a basal expression of AMPs to prevent bacteria from entering through the barrier and establish an infection. Studies have shown that *Shigella* species have the ability to downregulate the expression of human cathelicidin (LL-37) and human beta defensin 1 (hBD-1) through plasmid DNA to evade the immune survival at the first line of defense[107], while other studies reported that secreted proteins from the *Shigella* bacteria mediated the downregulatory effect [108]. As a proof of concept, *Shigella* was found to downregulate CAP-18 (the cathelicidin ortologue in rabbits) in the large intestines in a rabbit model[109]. More studies have demonstrated a downregulation of cathelicidin by *Vibrio cholerae* and *E. coli* in the large intestines through cholera and labile toxins, respectively[110]. In addition, it has been found that *Neisseria gonorrhoeae* downregulate LL-37 in a human cervical epithelial cell line[111].

Currently known inducers of AMPs in humans include vitamin D_3, which is a potent inducer of the *CAMP* (cathelicidin antimicrobial peptide) gene expression[13]. This induction has been studied *in vitro* and *in vivo*, where it is specific through gene responsive elements in the vitamin D_3 receptor (VDR) in primates, an element inserted late in evolution. For this reason, rodent cathelicidin genes do not respond to vitamin D_3 and rodents have therefore not been candidates for *in vivo* experiments studying vitamin D_3 -mediated effects on cathelicidin-expression[112,113].

In the gut, the microbiota, the epithelial cells and immune cells exist in a homeostatic system, which in the healthy state prevent pathogenic bacteria to invade the body and promote the normal flora to thrive. This homeostatic system involves the release of metabolic products such as butyrate (a short chain fatty acid) from the normal flora, which results in the production and secretion of AMPs. Butyrate is a known inducer of LL-37 and has been studied along with phenylbutyrate (PBA) as potential drugs for host directed therapy[114]. Butyrate and PBA are histone deacetylate inhibitors (HDACi) that can regulate gene expression through histone modifications[15,115].

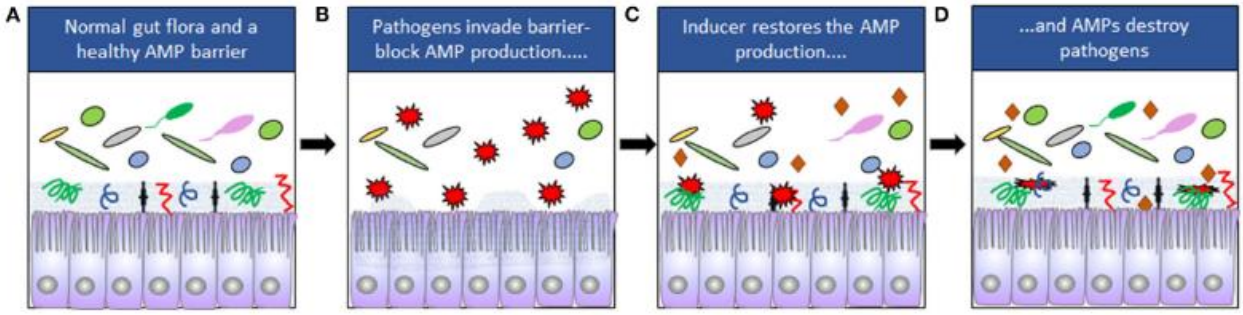


Figure 4. Host-directed therapy. *A) The normal gut microbiota is dependent on expression of AMP's. Mucins are indicated by the grey zone at the apical site of the cells and AMPs by symbols. B) Some pathogens (red) can downregulate the expression of AMPs and disrupt the epithelial barriers. C) Downregulation can be counteracted by inducers. D) The pathogens are eliminated when AMP expression is restored. Figure reused with permission from the publishing journal*[100].

The two inducers, vitamin D_3 and PBA, have been studied in combination, where both *in vitro* and *in vivo* studies have revealed a synergistic induction of *CAMP* gene expression in the presence of both vitamin D_3 and PBA[15,116,117]. Another potent inducer was found through screening of a chemical library to search for the induction of AMPs and the HDACi Entinostat, an approved drug used in combination with chemotherapy of cancer, was identified. Entinostat was effective in a rabbit-model for *Shigella* spp and *Vibrio Cholera* infections. However, the drug was found to be slightly toxic at higher concentrations[118,119]. Therefore, the search for additional AMP-inducers have continued and our research-group has identified a novel class of inducers (aroylated phenylene diamines), where the compound HO-53 was selected for further studies[120].

Pathogens such as *Mycobacterium tuberculosis* (Mtb), *Salmonella* and *Legionella* have evolved and acquired the ability to circumvent or block autophagy inside cells[121]. Autophagy is an important cellular defense mechanism to eliminate phagocytosed pathogens, and serves as an interesting target mechanism for host directed therapy[38]. Vitamin D_3 has been shown to activate autophagy as well as inducing the autophagy genes (ATG-5 and Beclin-1). The combination of vitamin D_3 and PBA showed a further increase in autophagy activation[122,123].

The activation of autophagy is a delicate balance, where the beneficial effect appears to be pathogen specific. Mtb have the mechanism to specifically downregulate autophagy by blocking the fusion of the phagosome and lysosome within the cell. With this, the bacteria can survive intracellularly in human macrophages, which contributes to their virulence[124,125] Activating the autophagy has been linked to increased killing of Mtb[126]. In contrast, *Staphylococcus aureus* and *Pseudomonas aeruginosa* on the other hand can use autophagy to their benefit and cause disease, making the induction of autophagy a delicate mechanism to manipulate[127–129].

Another important mechanism for bacterial killing and clearance is the ROS production. ROS is used both during phagocytosis, is released as a response to pathogens, and has to be closely regulated to avoid self damage to the cells[29]. The induction of ROS-production in a controlled and local manner could be an interesting approach aas a part of host-directed therapy against bacterial infections.

A link between ROS and antibiotic susceptibility has been proposed, where the increase in ROS upon antibiotic treatment has been recorded after ciprofloxacin treatment, as well as the killing efficacy of antibiotics, such as aminoglycosides, fluoroquinolones and β-lactam anticiotics was shown to be decreased under anaerobic conditions. The link between ROS and the effects of antibiotics has been contradictory due to difficulties in measuring ROS.[130].

The concept of host-directed therapy as a way to fight MDR bacteria is a promising research topic that deserves increased attention, and will be further studied and discussed in this thesis.

2 AIM OF THE THESIS

The general aim of the thesis is to gather knowledge on the connection between the innate immune system and multidrug resistant bacteria, to study host-pathogen interactions and examine the effects of host innate effectors on resistance mechanisms in Gram-negative bacteria.

- To study the impact of cellular supernatants on the effects of antibiotics against multidrug resistant *K. pneumoniae* (**Paper I**)

- To evaluate the effect of novel inducers of innate immune effectors (AMPs, ROS and autophagy) in human macrophages to eliminate multidrug resistant *K. pneumoniae*. (**Paper II**)

- To evaluate the survival of ESBL and non-ESBL *E. coli* isolates against innate immune effectors *in vitro* and *in vivo* (**Paper III**)

3 METHODOLOGICAL CONSIDERATIONS

Here I will review some methodological aspects in relation to my studies. I will focus on the isolates selected for the studies and the main methods used, including bacterial survival, cell culture and infections *in vitro* and *in vivo* as well as plasmid transfer.

In **Study I-III**, clinical isolates were used (*E. coli* and *K. pneumoniae*), isolated from bloodstream infections, retrieved from blood cultures and analyzed at the Karolinska University Laboratory in Stockholm (2013-2014). The isolates were characterized for antibiotic susceptibility and β-lactamase expression. In **study III**, a collection of randomly selected ESBL and non-ESBL *E. coli* isolates was used.

Bacterial survival was evaluated with two methods in **Study I-III**, with a colony forming unit assay (**CFU assay**) and with **Bioscreen C** survival curve generator. For both methods, a colony from a blood agar plate was cultured in the desired broth (LB or MHB broth) at 37°C and allowed to reach exponential phase. The bacterial suspension was then diluted to a working concentration (Optical density, OD_{600}) with appropriate conditions for each experiment in either a 96 well round bottom plate (CFU assay) or a Honeycomb plate (Bioscreen).

Whole blood/serum killing of bacteria was performed with a CFU assay in **Study III**. Blood was collected from healthy volunteers in either a heparin tube (whole blood) or coagulation tube (serum). The whole blood and serum were diluted in broth to a final concentration, and bacteria suspension added to the well. A **CFU assay** was then performed and survival was analyzed with viable CFU counts.

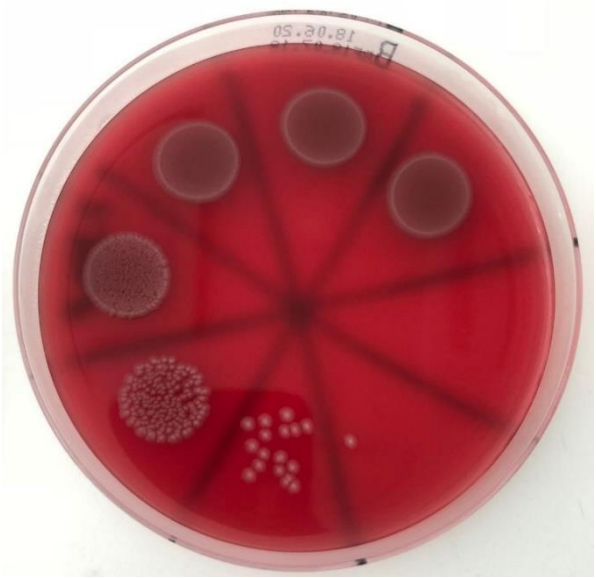


Figure 5. CFU counting. *When bacteria had been treated with the selected treatment (drugs, blood components, inducers), the suspension was diluted tenfold to produce 8 dilutions including the initial suspension. Each dilution was plated on a blood agar plate divided into 8 parts to visualize the logarithmic differences in bacterial survival. The lowest dilution with countable colonies (10-50 CFUs) was used for CFU counting. A picture from the author's experiments.*

Statistical analysis of viable CFU counting can be complex due to the extreme range of CFU/ml between conditions and experiments. To account for this, the CFU/ml were log transformed (where 0 CFU counts were considered as 1 CFU to allow for the logarithmic transformation). This transformation was followed by an ANOVA or t-tests for normally distributed conditions, or Mann-Whitney and Kruskal-Wallis in the case of ranked test.

To study the effect of AMP inducers in **Study II**, primary cells (peripheral blood mononuclear cells) were isolated from buffy coats and cultured into macrophage like cells in the presence of human macrophage colony stimulating factor (M-CSF), directing the differentiation towards an M1 state (pro-inflammatory) (phenotype confirmed via flow cytometry by a collaborating group)[131]. The cells were chosen for their phagocytosis and differentiation potentials. While human monocyte cell lines are available (THP-1 cells), the primary macrophages were considered more relevant for the purpose of AMP induction. The cells were analyzed through bacterial infections, Real-time PCR (RT-PCR) and Western blot analysis.

The primary macrophages were used as a model for a **gentamicin protection assay** in **Study II**, where the cells were stimulated with the treatment and infected with either a WT or MDR *K. pneumoniae*. The isolates used for the infection were susceptible to the antibiotic gentamicin, which is known to have no or limited intracellular activity. Gentamicin was added to the macrophages to kill extracellular bacteria, allowing for evaluation of live intracellular bacteria through lysing of the cells, plating and counting viable colonies. In **study II**, the use of vitamin D_3 as an inducer required the macrophages to be from humans (AMP induction and responses alter between species), however in **study III** the need for a human cell line was not present and the mouse macrophage cell line RAW 264.7 was cultured and used in the **gentamicin protection assay**. Using a cell line eliminates the need for live donors and can provide rapid and reliable results without donor variation.

In **study I**, supernatants were collected from HT-29 cells, a cell line established from human colon cancer cells[120]. The cells were selected for the relevance to intestinal infections and were only used to produce supernatants, eliminating the need to establish a primary cell model. The supernatants were further used in the search for an endogenous β-lactamase inhibitor.

In **study III,** a **Zebrafish embryo infection model** was utilized for *in vivo* experiments. The zebrafish have emerged as an exceptional tool to study the immune system. The optical transparency of the fish, the accessibility to visualize the organs and the rapid development from embryo to fish gives this model a great advantage. The jump from *in vitro* cell culture to *in vivo* mouse studies is very high, and the zebrafish model reduces this jump in both time, cost and accessibility.

The generation of **genetically altered lineages** is highly successful in the zebrafish, where gene inactivation and transient gene knockdown has proven a useful tool to gain information into genetics and cell biology. The model has been used extensively to research hematopoietic and lymphoid development, where gene expression data from microarrays can be used to study

differences in gene expression between wild type and mutant embryos. This research is crucial in the understanding of genetic conditions and genetic diseases in humans[132].

The zebrafish is an efficient tool for **drug discovery**, having the advantage of phenotype-based screening, where more variables such as pain, sedation, tumor metastasis, vascular tone and gut motility of a disease can be recorded. The model also provides a very early insight into a drug's toxicity on different organs, which is limited in cell-based assays. The relevance of an animal model to human physiology is an important factor to consider. There are always limitations on each animal model used, however recent studies have shown that 82% of disease-associated targets in the zebrafish are shared with humans, where the zebrafish physiology is highly conserved[133].

The focus of this thesis is based on the innate immune system. The zebrafish have been found to exhibit **innate immune defenses** the resemble the human innate immune system. They have circulating erythrocytes, neutrophils, eosinophils, lymphocytes, macrophages, dendritic cells, exhibiting a highly conserved hematopoietic system[134].

The zebrafish express TLR's on the surfaces of macrophages and dendritic cells and recognize conserved pathogenic traits through PAMP's[132]. **The complement system** of the zebrafish has been studied extensively, showing all the homologs of the mammalian complement components being present. This suggests that the complement system in the zebrafish is similar to the mammalian/human complement system, both structurally and functionally[135]. Finally, **antimicrobial peptides** have been discovered in the zebrafish[136], emphasizing the relevance of this animal model to the research on innate immune defenses and host directed therapy.

In **study III**, the method of zebrafish microinjections was utilized, where bacteria were injected into the embryos and the survival of the embryo/fish recorded over 5 days. This method does not allow for pre-treatment of the fish since the embryo can only be injected once due to the embryos fragility, however the method shows great promise when studying different bacteria in the fish, or the effect of genetic modulation to the innate immune defenses.

In **study I** and **study III**, a bacterial **trans-conjugation** method was used to transfer plasmids from one bacterial strain to another. Plasmid trans-conjugation requires a defined selection for each strain, which can be difficult to achieve with clinical isolates. For this method, a two-step trans-conjugation was performed, moving the resistance plasmid from the clinical isolate into an intermediate strain with a double selection. This method constitutes of a donor strain containing VIM-1 (**Study I**) or ESBL (**Study III**), an intermediate strain, and a final recipient strain. Importantly, the donor strain is required to be susceptible to chloramphenicol, and resistant to the selection antibiotic (AB) (e.g ampicillin). The intermediate strain was susceptible to the selection antibiotic, resistant to chloramphenicol, and dependent on 2,6-diaminopimelic acid (DAP)(*Δdap*). For the first step of the plasmid transfer, using a selection antibiotic, DAP and chloramphenicol selects only for the intermediate strain that has acquired the plasmid (with a DAP (-) control showing no growth). For the second step of the transfer, a final recipient strain, susceptible to the selection antibiotic was trans-conjugated with the intermediate strain, selecting the final product with the selection antibiotic only without DAP,

allowing the growth of the final recipient that has acquired the plasmid only (no DAP, with selection antibiotic) (Figure 6)

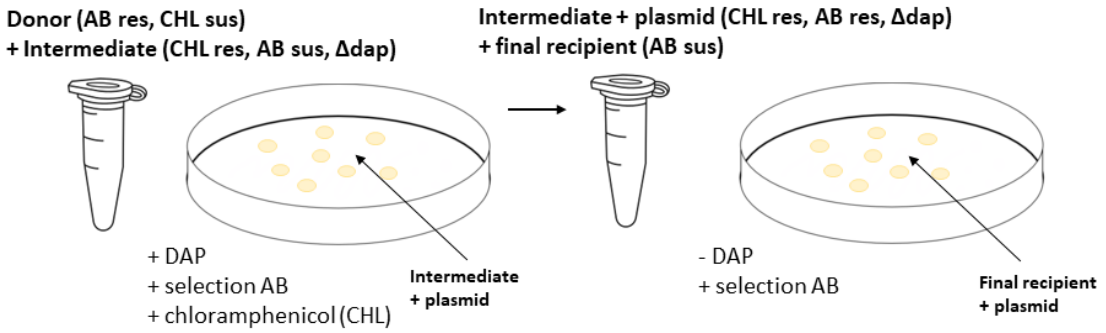


Figure 6. Plasmid transconjugation selection. *Strains with different selection markers were used to form the final product of a final recipient strain containing an ESBL/VIM-1 plasmid. The donor strain (clinical isolate) conjugated with an intermediate strain (resistant to CHL, DAP dependent and AB susceptible), resulting in a the intermediate strain containing the plasmid (selection AB killing intermediate without plasmid, chloramphenicol killing the original donor). In the second step, the intermediate+plasmid strain (CHL resistant, AB resistant, DAP dependent) is conjugated with a final product (AB susceptible) and selected with AB only (no DAP), allowing for the growth of the final recipient that has acquired the resistance plasmid. Selections were performed with single selection controls.*

To avoid the occurrence of false positive growth, all steps of the trans-conjugation included a single or zero selection controls. If the intermediate strain with the plasmid grew in the absence of DAP, a false positive selection had occurred. This was followed closely, and the plasmid acquisition was confirmed with a colony PCR using primers for the transferred plasmid on all strains involved in the selection (donor strain, intermediate strain, intermediate strain with the plasmid, the final recipient strain and the final recipient strain with the plasmid).

To conclude, the main methods used in **Study I-III** have been described in detail, including the rationale to why these cells and methods were chosen. For further detailed methods, see **papers I-III**.

4 RESULTS AND DISCUSSION

(for more detailed information, see **papers I-III**)

THE IMPORTANCE OF THE MICRO- ENVIRONMENT FOR THE EFFECTS OF ANTIBIOTICS DURING INFECTION

Study I was concluded in the published paper "*Klebsiella pneumoniae* expressing VIM-1 metallo-β-lactamase (MBL) is re-sensitized to cefotaxime via thiol-mediated zinc chelation". The study was focused on searching for cellular factors with the capacity to inhibit carbapenemases. Antibiotic resistance in bacteria is normally measured in a standardized nutritious experimental environment, which is important to maintain consistency and accuracy between different laboratories[137]. The general conditions under which antibiotic resistance is evaluated is however not representative of the local host environment where the infection is established[98].

In this study, *K. pneumoniae* isolates expressing different β-lactamases were screened for susceptibility against cefotaxime when cultured in the presence of cell culture supernatants from colon epithelial cells. The cells produce and metabolize various components that can influence bacterial survival and antibiotic susceptibility. Two of the isolates tested (both expressing VIM-1 metallo-β-lactamases) showed increased susceptibility to cefotaxime, while clinical isolates expressing NDM, KPC and OXA did not. Different antibiotics were tested together with the supernatant, however a restored susceptibility was detected only for cefotaxime and ceftriaxone (third-generation cephalosporins). Given that the VIM-1 enzyme is a zinc dependent metallo-β-lactamase, the role of metal chelation was studied by exchanging the supernatant for RPMI cell culture media supplemented with EDTA. By using EDTA treatment, as well as metal supplementation in the supernatant, it was concluded that zinc chelation was an important factor for the observed resensitization effect.

To study if the supernatant contained a zinc chelation component, or if the cause for this effect was the lack of available zinc, the supernatant was subjected to various filtration- and chemical treatments. The active component was identified as a small (<3kDa), hydrophilic compound.

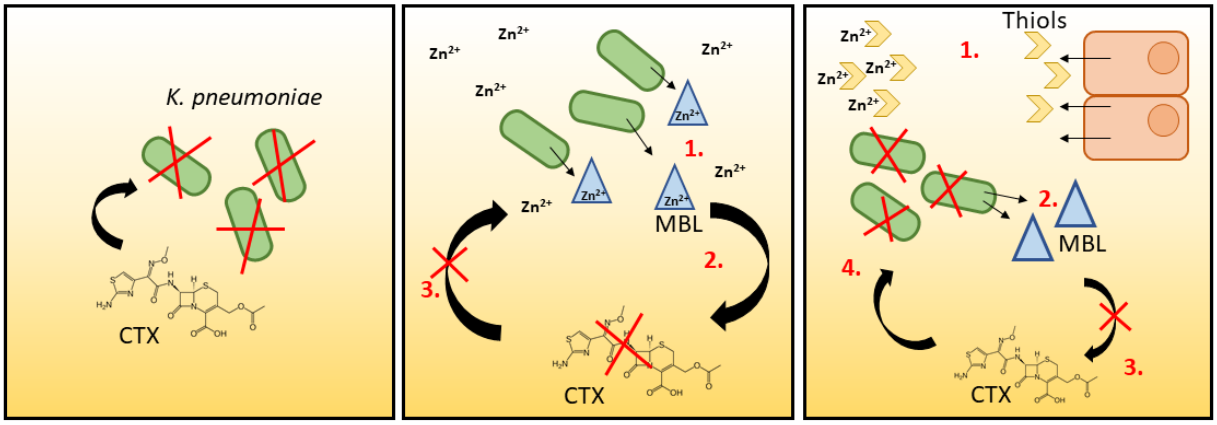
Finally, with the use of L-cystine and L-cysteine, and substituting the cell culture media to DMEM without L-cystine, it was concluded that the cells convert L-cystine (from RPMI/DMEM media) to L-cysteine (generating free thiols) that chelate available zinc, and thus prevent the VIM-1 enzyme from hydrolyzing the antibiotics. This was confirmed further by the addition of glutathione (both oxidized and reduced form), and the use of L-cystine that emits a fluorescent signal when reduced to L-cysteine. Blocking the thioredoxin pathway resulted in a dose dependent decrease in the conversion of L-cystine to L-cysteine**.**

To study if the genetic background of the bacteria was responsible for the observed resensitization, a two-step plasmid transconjugation was performed on the VIM-1 producing strain (AO15200). The plasmid was transferred to two neutral backgrounds (*E. coli* ATCC 25922 and MG1655). The *E. coli* strains carrying the VIM-1 plasmid exhibited the same re-

sensitization to cefotaxime in the presence of L-cysteine, confirming that the zinc chelation ability of thiols inhibited the activity of the enzyme.

Gram-negative bacteria are a common cause of urinary tract infections that can lead to bacteremia and sepsis[138,139]. Urine is a thiol rich fluid and the growth of the bacteria was studied in urine samples from healthy volunteers[140]. When the supernatant was replaced with diluted urine, the VIM-1 producing bacteria was susceptible to cefotaxime. When the urine was fractionated, the effect was observed in the same fractions where L-cysteine was found, confirming that the thiols in urine were a contributing factor to the re-sensitization to cefotaxime. Re-introducing zinc into the system regained the resistance and growth of the bacteria.

MBLs require zinc to hydrolyze β-lactam antibiotics. Given the data from **Study I** on VIM producing strains the same should apply to other MBLs. The three common MBLs include VIM, IMP and NDM, where IMP expressing strains were not accessible for this study, but NDM strains were screened. However, NDM-producing clinical isolates are known to carry other resistance plasmids (such as ESBL), which would counteract the effect observed in the supernatant. When a lab strain expressing only NDM resistance was acquired, a similar resensitization effect against cefotaxime was observed in the presence of L-cysteine.



Cefotaxime (CTX) directly kills *K. pneumoniae*

1. *K. pneumoniae* produces MBL
2. MBL binds Zn^{2+} to hydrolyze CTX
3. *K. pneumoniae* is not killed by CTX

1. Cells produce thiols that chelate Zn^{2+}
2. *K. pneumoniae* produces MBL
3. MBL cannot bind $Zn^{2+}$and does not hydrolyze CTX
4. *K. pneumoniae* is killed by intact CTX

Figure 7. A graphical summary of Study I. *The main results from Study I have been compiled into a graphical summary.*

INDUCING THE INNATE IMMUNE SYSTEM TO ELIMINATE MDR BACTERIA

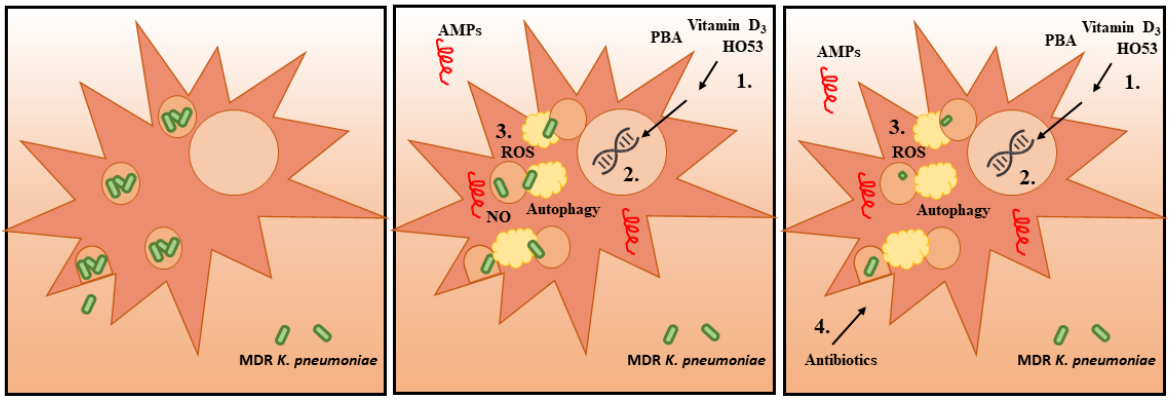
In **Study II**, we hypothesized that induction of innate immune effectors in human macrophages would contribute to intracellular killing of MDR bacteria. To that end, primary macrophages, differentiated to an M1 pro-inflammatory phenotype[141], were treated with known inducers of AMPs for 24 h before the addition of both MDR and antibiotic susceptible *K. pneumoniae*. All inducers: PBA, vitamin D_3, PBA + vitamin D_3, HO53 and HO53 + vitamin D_3, reduced the intracellular survival of both *K. pneumoniae* strains. Notably, the inducers did not have a direct killing effect on the bacteria.

Antibiotics that were found ineffective against the MDR *K. pneumoniae* were added to the cells during the infection to study the potential of further enhancing the killing of the bacteria. The antibacterial effect of azithromycin, cefotaxime, ciprofloxacin and fosfomycin was found to be enhanced with different combinations of inducers. Azithromycin activity was enhanced when the cells had been treated with PBA, vitamin D_3 + PBA, HO53 and HO53 + vitamin D_3. Similarly, cefotaxime activity was enhanced with PBA + vitamin D_3, HO53 and HO53 + vitamin D_3. Ciprofloxacin activity was enhanced together with PBA, + vitamin D_3, HO53 and HO53 + vitamin D_3, while fosfomycin had less potent killing enhancement with the inducers, showing a difference only with the combination of PBA + vitamin D_3 and HO53 + vitamin D_3. This suggests that pretreatment of human macrophages with inducers of innate effectors has the potential to sensitize the cells to allow for enhanced killing of MDR bacteria. In addition, some conventional antibiotics have been shown to directly activate innate effector mechanisms in host-cells[100]. This phenomenon likely contributed to the enhanced intracellular killing observed in this project. Host directed therapy exploits alternative and more diverse ways to eliminate pathogens, whereas monotherapy with conventional antibiotics may be ineffective or lead to rapid development of resistance.

To dissect which effector systems were involved in the enhanced bacterial killing, transcriptional changes of a number of key genes were analyzed. Notably, the expression of the *CAMP*-gene, encoding LL-37 and HBD-1 was induced by PBA, vitamin D_3 and HO53. The inducers activated other AMPs, such as HBD-2 and HBD-4, and the oxidative stress genes nitric oxide synthase-2 (*NOS2*), dual oxidase 2 (*duox2*), the catalase encoding gene *CAT* and glutathione peroxidase 3 (*GPx-3*). Finally, the inducers activated autophagy related genes (*atg5* and *atg12*), indicating a diverse innate immune response by the inducers including antimicrobial peptides, reactive oxygen/nitric species and autophagy.

The inducers were originally selected based on the induction of the *CAMP* gene encoding LL-37, however more diverse effects on innate immune effectors were observed. To study to which degree LL-37 was involved as a contributing factor in the outcome of the bacterial killing, LL-37 was silenced in the macrophages with siRNA. Bacterial growth was restored when LL-37 was silenced, despite the presence of the inducers, PBA and/or vitamin D_3, emphasizing the importance of LL-37 for bacterial killing. HO53, an HDACi, did not show the same effect on bacterial growth, suggesting a different mechanism of action for this compound within

macrophages **(Figure 5)**. Next, we used macrophages deficient in reactive oxygen species production from a patient with a genetic mutation in the CYBB gene, and healthy macrophages where the CYBB-gene was silenced. Interestingly, an impaired intracellular killing capacity with PBA, vitamin D_3, PBA + vitamin D_3, HO53 and HO53 + vitamin D_3 was observed in these cells, indicating that macrophages indeed require ROS production to inhibit bacterial growth **(Figure 6)**. Finally, the importance of autophagy was confirmed using an autophagy inhibitor, resulting in the same trend of increased bacterial growth and abolished effect of the inducers **(Figure 7)**. The results from **Study II** underline the potential to induce innate immune responses, alone or in combination with conventional antibiotics, to combat infections caused by MDR *K. pneumoniae*.



MDR *K. pneumoniae* is phagocytosed by macrophages and can survive intracellularly

1. Treatment with innate immune inducers
2. Induction of AMP production, ROS/NO production and activation of autophagy
3. Increased intracellular killing of *K. pneumoniae*

1. Treatment with innate immune inducers
2. Induction of AMP production, ROS/NO production and activation of autophagy
3. Increased intracellular killing of MDR *K. pneumoniae*
4. Antibiotic treatment induces further killing of intracellular MDR *K. pneumoniae*

Figure 8. A graphical summary of Study II*. The main results from Study II have been compiled in a graphical summary*

ESBL-PRODUCING *E. COLI* ARE MORE SUSCEPTIBLE TO INNATE EFFECTORS THAN NON-ESBL *E. COLI*

In **Study III** we hypothesized that ESBL-producing *E. coli* would be more susceptible to innate immune effectors than non-ESBL E. coli. The rationale was based on the fact that immunosuppression is a risk-factor for invasive infections with MDR bacteria in general. However, the underlying mechanisms are poorly understood. To test this hypothesis, *E. coli* isolated from bloodstream infections were analyzed in the presence of innate immune defenses. The isolates were randomly selected from a larger collection and categorized into ESBL-producing isolates or non-ESBL isolates, and the susceptibility to innate immune defenses were studied (serum, whole blood, AMPs, intracellular killing in macrophages). A total of 60 isolates (out of n=142) fit the criteria; *E. coli* ESBL producing (n=30) or non-ESBL isolates (n=30). First, the isolates were tested for survival in human serum and ESBL producing isolates survived to a significantly lower degree compared to non-ESBL isolates.

From the collection of 60 *E. coli* isolates, 10 isolates were randomly selected from both groups, resulting in a selection of 21 isolates (10 ESBL, 10 non-ESBL and 1 ATCC reference strain), which were chosen for further analysis. Again, the ESBL isolates exhibited significantly lower survival in human whole blood and serum, and heat inactivation of the serum abrogated the killing. This suggests that the complement system is an important contributor to the difference of survival between ESBL and non-ESBL producing isolates in serum. The intracellular elimination of the pathogens was also studied with RAW 264.7 macrophages, where ESBL producing isolates had lower intracellular survival compared to non-ESBL isolates.

The cost of fitness associated with antibiotic resistance has been studied mostly in the form of internal fitness of bacteria, such as growth rate[85]. The results from **Study III** however suggest that there is a biological cost connected to the ESBL phenotype in the form of increased susceptibility against innate immune responses. The reason for this cost could be related to the presence of the ESBL plasmid itself, or connected to the genetic background (chromosomal genes) of the bacteria. Both alternatives were explored by 1) transferring the ESBL plasmid from a clinical isolate to a clean background via plasmid trans-conjugation, and 2) analyzing the genomes of the different isolates and comparing non-ESBL isolates to ESBL isolates.

A trans-conjugation setup was utilized, moving the ESBL plasmid from a clinical isolate, through an intermediate selection strain, and finally into an ATCC 25922 lab strain. The results showed no significant difference between the ATCC 25922 strain and the ATCC-ESBL conjugate in either serum or whole blood, suggesting the plasmid transfer did not alter the survival of the bacteria**.** The 21 isolates were sequenced and genomic data was compared with regard to: serotype, sequence type, antibiotic resistance and virulence factors. With a small cohort of 21 subjects, correlation studies were difficult to perform, however a difference was observed in the serotype distribution of the *E. coli* isolates where the two groups showed a distinct separation in O and H antigens.

The increased susceptibility to serum was further studied in an *in vivo* zebrafish embryo model. Embryos injected with RS020 (non-ESBL) had lower survival compared to RS006 (ESBL), which confirmed the results that the ESBL isolates were eliminated more efficiently than non-ESBL isolates *in vivo*. Additionally, the transconjugants were studied in the zebrafish model, and no significant difference in survival of the embryos injected with the ATCC 25922 strain and ATCC-ESBL transconjugant was observed.

The data from **Study III** suggests that increased serum susceptibility of ESBL producing isolates is dependent on the genetic background and potential structural differences in the bacteria, and not the ESBL plasmid acquisition *per se*. The study was limited to a small number of clinical isolates and expanding the sequencing and genetic analysis to a larger collection could most likely provide better information.

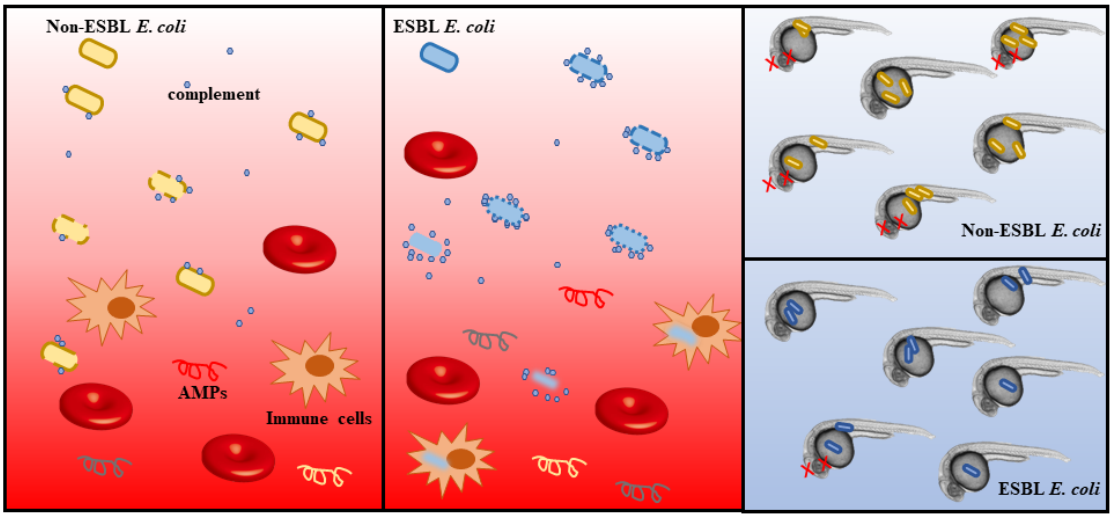


Figure 9. A graphical summary of Study III*. The main results from Study III have been compiled in a graphical summary*

Together, **Study I-III** highlight the importance of the tissue micro-environment and innate immune defenses for the pathophysiology and treatment of multidrug resistant bacteria. Studying the host-pathogen interactions and environment could be the key to solving the increasingly difficult problem of antibiotic resistance, where according to **Study I,** the contribution of the host is an important consideration. **Study II** addressed the possibility of using inducers of innate immune defenses (AMPs, ROS, autophagy etc), which could be a promising alternative or addition to current treatment options. Finally, **Study III** presents the differences between ESBL producing and non-ESBL *E. coli* in the presence of blood and its components underlying the contribution of the host response to invading pathogens.

Finding ways to regulate the environment at the site of infection and the availability of metabolites, nutrition and metals could lead to the discovery of new and improved β-lactamase inhibitors.

5 FUTURE PERSPECTIVES

Past research on antibiotic resistant bacteria has mainly been focused on the direct inhibition of the resistance mechanisms, as well as finding new antibacterial compounds. The search for inhibitors of MBLs has been ongoing for years, however no clinically available inhibitors are currently accessible. A novel compound, ANT431, has been developed as an MBL inhibitor[142]. The inhibitor was active against purified NDM-1 and VIM-2 enzymes and in combination with meropenem on NDM-1 *Enterobacterales*, however, the activity had limited range against different MBLs. The limited range of MBLs inhibited by this compound will most likely prevent the inhibitor to become a clinical candidate, but can be used as a model for further research[143]. Multiple inhibitors for serine-β-lactamases have been discovered, and research is still ongoing in developing new compounds. A new inhibitor VRNX-5133 has been developed, showing activity against both serine-β-lactamases, as well as VIM and NDM in *P. aeruginosa*[144]. The development of novel β-lactamase inhibitors is constantly ongoing, however the use of monotherapies might become outdated, since resistance to both β-lactams and β-lactamase inhibitors is emerging rapidly[143].

Targeting the host-pathogen interaction could be an alternative option to combat antibiotic resistant bacteria. Accessing the problem from diverse directions, along with the discovery of novel antimicrobials and inhibitors, is a promising alternative for future research and treatment[100]. More diverse ways to target the host-pathogen interactions have been proposed, such as the neutralization of virulence factors, blocking epithelial adherence and biofilm formation, the use of monoclonal antibodies against key pathogens, toxin neutralization and more specific methods to alter this interaction during infection, that focus on suppressing the growth and survival of the bacteria, leaving them harmless instead of directly killing the pathogen[145]. For this to become an alternative, methods of antibiotic susceptibility need to evolve in the direction where the host environment is taken into account, determining minimum inhibitory concentrations in assays that are relevant for *in vivo* infections[99].

The use of omics and computer simulations is becoming increasingly accessible and useful in many fields of biology. Sequencing technologies are more cost efficient and the generation of whole genome sequencing data has become a standard technique. The gradual development in omics (such as transcriptomics, metabolomics and proteomics) allows for a broader use of datasets, combining different methods to determine structure, function and potential antimicrobial activity of compounds. The interaction between pathogens and antimicrobials is complex and can include many different targets and pathways. Computational models will likely become a leading technological method in the host-pathogen interactions and the discovery of novel antimicrobials[146,147].

6 CONCLUDING REMARKS

The studies compiled in this thesis have together highlighted the importance of considering the host cellular environment, metabolism and immune responses in the fight against MDR infections. Host-cells regulate both the extracellular and intracellular environment through metabolism and maintain a controlled tissue micro-environment. The availability of nutrients, metabolites and essential metals can limit (or sustain) the bacterial survival and potential of infection. Targeting the antibiotic resistance mechanisms directly by limiting the availability of extracellular components needed by the bacterium (**Study I**) or inducing the cellular response to infection could be key factors in managing the infection (**Study II**). It is vital to gain more information if the host response can possibly distinguish between non-resistant and resistant pathogens. Given the results that MDR strains may be more sensitive to innate immune defenses (**Study III**), the development of host directed therapy emerges as a strong and promising alternative or addition to mono-therapeutic methods. The fields of pharmacology, immunology and microbiology have remained separate, while research areas in oncology, for example, have evolved together with immunology. This has resulted in a breakthrough in cancer therapeutics and sets an example how merging fields can improve research and communication[145]

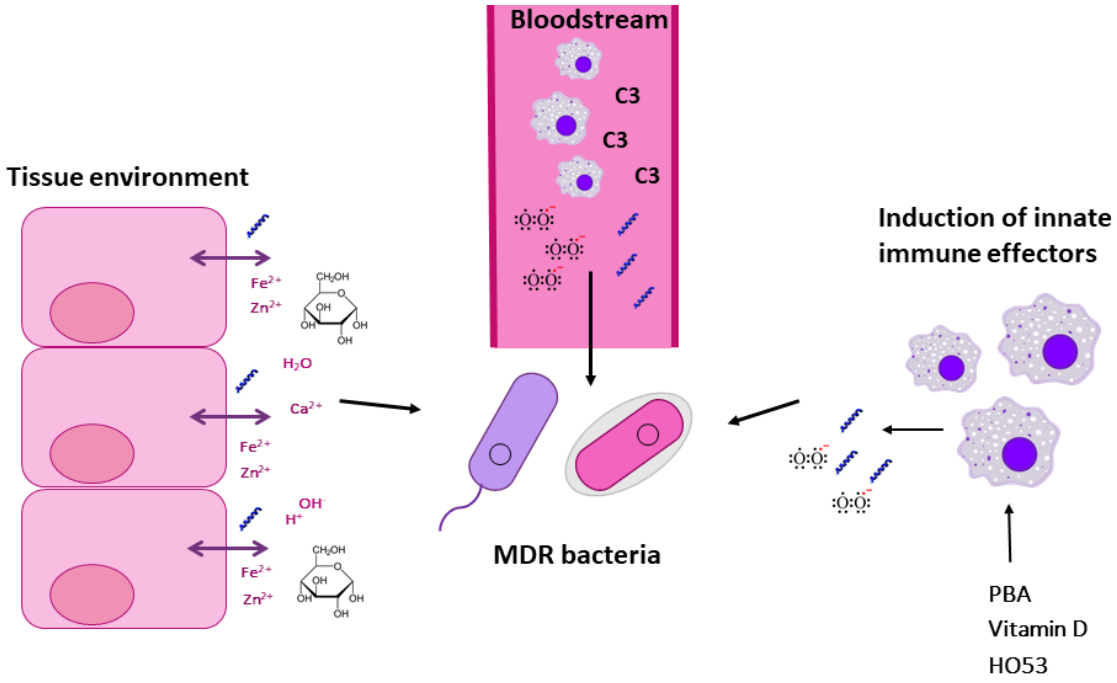


Figure 10. Study I-III compiled in a schematic picture. *Study I explored the role of tissue environment in the process of antibiotic resistance. In Study II, inducers of innate immune effectors were used to reduce intracellular bacteria survival within macrophages. Study III showed that the innate immune system affects non-resistant and resistant bacteria differentially, all together emphasizing the importance of developing host directed therapy treatments.*

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